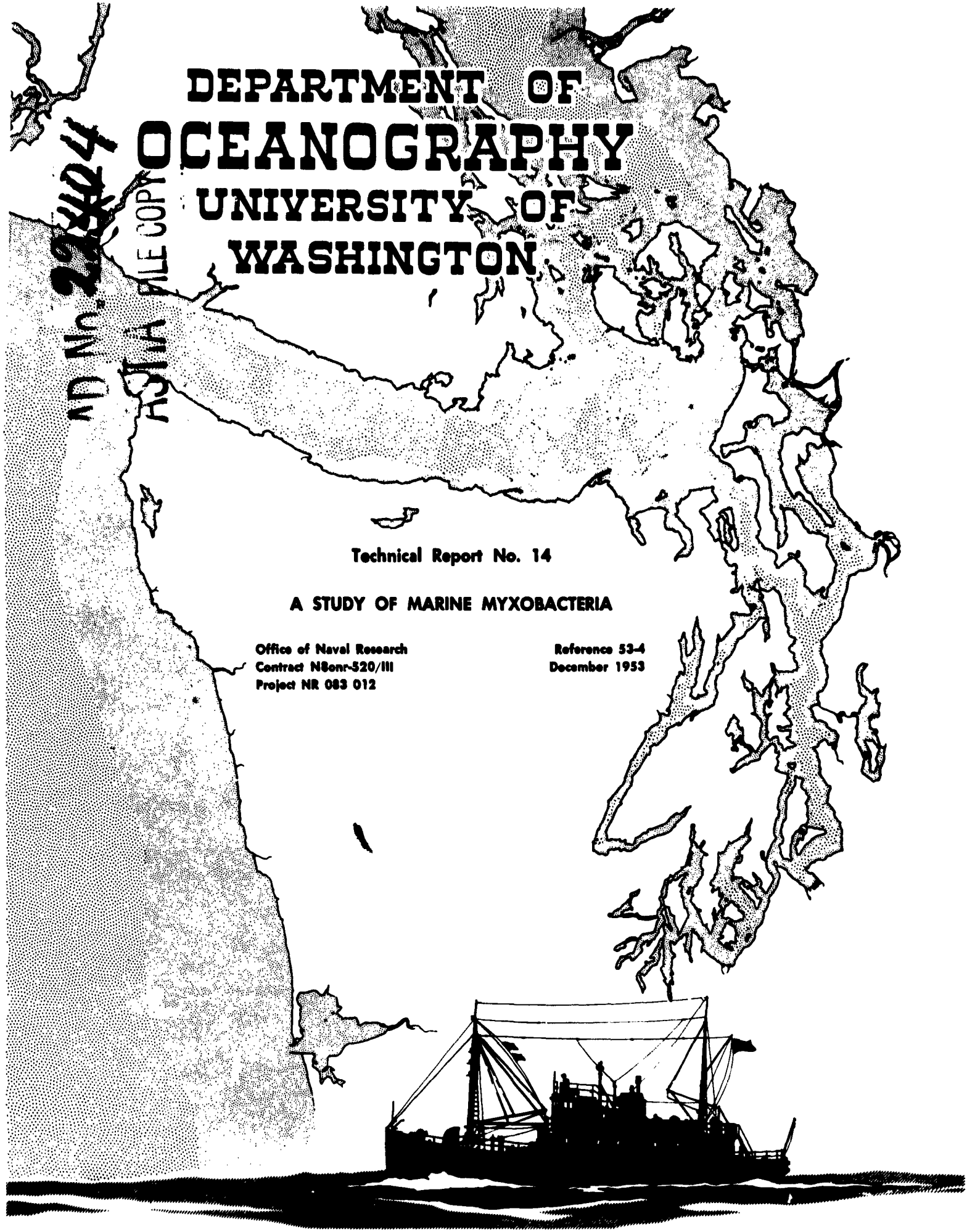
DEPARTMENT OF OCEANOGRAPHY
UNIVERSITY OF WASHINGTON

Technical Report No. 14

A STUDY OF MARINE MYXOBACTERIA

Office of Naval Research
Contract N8onr-520/III
Project NR 083 012

Reference 53-4
December 1953



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UNIVERSITY OF WASHINGTON DEPARTMENT OF OCEANOGRAPHY
(Formerly Oceanographic Laboratories)
Seattle, Washington

Reference No. 53-4

A STUDY OF MARINE MYXOBACTERIA

by

Theodore J. Starr and Erling J. Ordal

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Richard H. Fleming
Executive Officer

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A STUDY OF MARINE MYXOBACTERIA

STATEMENT OF PROBLEMS

This investigation is concerned with the study of myxobacteria from the marine habitat. These microorganisms, referred to as "slime bacteria," do not normally develop on the culture media commonly employed in the bacterial analysis of sea water.

There is little published information on aquatic types of myxobacteria, and the majority of our information on myxobacteria has come from the study of terrestrial forms. These are best known because of the occurrence of complex life cycles, and because many of them are capable of utilizing high molecular compounds such as cellulose and chitin. However, studies carried out at the University of Washington by one of us (E. J. Ordal) have shown that myxobacteria are common in the aqueous habitats. In fresh water a wide variety of well-defined saprophytic and parasitic myxobacteria have been found. Some of these cause serious diseases in fish. Preliminary studies have shown that myxobacteria are also present in sea water, particularly in association with plankton and other living forms. In addition, myxobacteria are often found in samples of aged sea water which have been stored in the dark. This information, coupled with the well-known habit of development on surfaces, suggested that myxobacteria might be concerned with the mineralization of relatively stable organic compounds such as found in animal and plant detritus.

Marine myxobacteria were sought in samples of sea water and sediments from Fort Orchard. In addition, several samples of aged sea water from the Beaufort Sea were selected for study.

It was found necessary to devise special methods and media for the isolation of myxobacteria. Also, studies on the physiology of marine myxobacteria were carried out as a prerequisite to obtaining knowledge of the function and distribution of myxobacteria in the sea.

A STUDY OF MARINE MYXOBACTERIA

INTRODUCTION

The group of organisms which constitute the present-day order Myxobacteriales was recognized in the late nineteenth century. Fundamental knowledge concerning these bacteria has accumulated slowly and is sadly lacking if one considers their widespread abundance and the considerable length of time which has elapsed since the first description of a member of this group in the early eighteen hundreds.

The apparent lag or dormancy of interest in the myxobacteria may be accounted for if one considers that their initial descriptions were made by the cryptogamic botanist rather than the bacteriologist, and that pure culture study was hampered by lack of suitable isolation techniques. Furthermore, the group did not include the imperfect myxobacteria until recently (Stanier, 1940), and hence was limited to the higher fruiting forms. In addition, the peculiar morphological traits of the recognized species aroused the interest of the morphologist rather than the biochemist or the physiologist. This latter fact is evidenced by the morphological definition and separation of the order Myxobacteriales into families, and by the significant absence of biochemical and physiological differentiation of all but a few species.

The major part of the literature pertaining to members of the order Myxobacteriales is concerned with morphological investigations of the terrestrial forms. Although little work has been published concerning the aquatic myxobacteria, a start has been made in this direction

by Ordal (1953) who has consistently observed and isolated myxobacteria from aquatic habitats. Otherwise the aquatic forms have been neglected to a great extent. They have been studied in this laboratory from both marine and fresh water environments. They have been observed in association with fish and aquatic plant life. Some members have been found responsible for epizootics in salmon. Furthermore, the etiological role of the aquatic myxobacteria accentuates the need for additional study of these forms. This investigation is an attempt to add to our knowledge of the marine members of the order, and to emphasize the need for further biochemical and physiological characterization as a supplement to the present morphological delineation of the order and members of the order.

The myxobacteria or slime bacteria are differentiated from the other bacterial groups by virtue of significant morphological properties which warrant this separation. However, certain traits are common to other groups of organisms, and this has caused some speculation as to the phylogenetic relationships of the myxobacteria. The following paragraphs describe these characteristics and briefly present their possible outside affinities to other microorganisms.

Myxobacteria form Gram-negative, rod-shaped, vegetative cells. In this respect they resemble some members of the order Eubacteriales. However, they multiply by constriction and lack a cell wall. They are flexible as are members of the order Spriochaetales. Flexibility is a major criterion for the initial identification of the aquatic myxobacteria. Individual cells may show a peculiar spinning motion, hanging from the surface of a drop by one end and rotating rapidly.

Vegetative cells may be tapered at one end but more often they are cylindrical. The length of cells varies considerably among different species and within a single clone. Cells as short as 3 microns and as long as 20 microns are common.

Organisms have been observed to move at a rate of 10 microns per minute. However, the mechanism of locomotion is still an unsolved problem. They have no flagella. This peculiar creeping motility is also characteristic of the Cyanophyceae and Beggiatoaceae.

All members of the order produce a reproductive unit termed a microcyst except for members of the family Cytophagaceae. The microcyst corresponds to the spore stage of members of the order Eubacteriales but differs in its mode of formation. It is considered to be formed by a shortening and thickening of the cell and is not formed within the cell as are endospores.

Fruiting bodies are formed by all myxobacteria with the exception of members of the genera Cytophaga and Sporocytophaga. These structures result from the combined efforts of the mass of vegetative cells acting as a unit. This concerted action of individuals toward a definite end is common to the Acrasieae which, however, have amoeboid cells (Thaxter, 1892).

Myxobacteria are widely distributed. They have been found in soils throughout the world (Breed et al, 1948), in compost and sludge (Singh, 1947), and parasitic on phytopathogens (Kononenko, 1937). Among the aquatic members of the order, one species has been described as parasitic on Cladophora fracta (Geitler, 1924). Other aquatic types

have been implicated in fish diseases (Ordal and Rucker, 1944; Garnjobst, 1945; and Borg, 1948). Two marine members have been studied by Stanier (1942). Humm (1946) described one marine form.

The purpose of this investigation was to study the marine members of the order Myxobacteriales. To this end, various methods were used to obtain marine samples. Isolation techniques were designed to obtain as wide a variety of representative species as possible. Diverse physiological and morphological types were then selected for further characterization of nutritional requirements, biochemical properties, and enzymatic activity.

COLLECTION OF MARINE SAMPLES

Samples of marine origin were obtained from a number of sources and by a multitude of techniques in an attempt to isolate as wide a variety of species as possible. Fresh samples were obtained from the Port Orchard and Port Madison Bay regions. In addition, samples of aged, stabilized sea water were obtained from Dr. C. A. Barnes. The latter samples which were 2 to 3 years old were obtained from the Beaufort Sea, located north of Alaska. Their past history is available. They represented composite samples of some 20 to 40 stations each, collected at graduated depths up to 3000 meters or more. The samples representing a depth range of 50 to 300 meters were selected for investigation after careful appraisal of their past history. They came from a depth in which the density increased very rapidly compared to the density above. Thus, the depths from which they were obtained acted as a sedimentary shelf for the water above.

The samples which were obtained from the Port Orchard and Port Madison Bay regions were collected by a variety of techniques and devices which included: the orange-peel bucket, a modified Emery-Dietz corer, plankton nets, a piston core sampler, and sterile water sampling bottles.

The oceanographic vessels BROWN BEAR and ONCORHYNCHUS were used for field trips, and the former vessel was equipped with the necessary bacteriological facilities.

ISOLATION OF PURE CULTURES

The question arose, as it does in any problem which involves the isolation of a representative cross section of a group of microorganisms, as to whether myxobacteria were present in each sample; and if they were present in a sample, was the medium used for isolation adequate to support their growth? Therefore, a wide variety of media and plating techniques were employed for each sample. It was felt that this approach would avoid the selective properties inherent to a single medium, and hence provide a more representative group of isolates.

Solid media were prepared and included the following additions to a sea water base and to a synthetic inorganic base: peptone, tryptone, beef extract, yeast extract, serum, glucose, and either gelatin or agar. Different concentrations and combinations were used. Subsequently, it was found that a sea water, 1.0 per cent agar basal medium which was supplemented with 0.05 per cent each of yeast extract, beef extract, and tryptone, would support best growth and the widest variety of microorganisms.

In addition to the solid media, enrichment media were used. They contained a sea water or inorganic base supplemented with either agar, chitin, cellulose, gelatin or soluble starch.

Samples were plated on the above solid media or seeded into the liquid enrichments. Myxobacteria were observed from all of the Beaufort Sea samples which were studied and from samples obtained at all of the six stations selected in the Fort Madison and Fort Orchard Bay regions. However, myxobacteria were not observed in all of the four to six different samples taken at each of the six stations. The top sediments and bottom water samples usually contained myxobacteria, whereas the bottom sections of the core samples and most of the surface plankton drags did not contain myxobacteria. Myxobacteria could be isolated from the Fort Orchard and Fort Madison samples by direct plating in contrast to the Beaufort Sea samples which required initial enrichments prior to plating.

On the basis of the [illegible] were observed during the course of this investigation, eight [illegible] selected for further characterization of properties since they [illegible] apparently different species and were representative of the [illegible] served. They were designated, respectively, BS1, BS2, BS3, [illegible], FO2, FO3, and FO4. The history of each isolate is discussed in the next section.

Stock cultures were kept on slants which contained sea water to volume, 1.0 per cent agar and 0.05 per cent each of beef extract, yeast extract and tryptone. The latter supplement will be referred to as cytophaga supplement, and the medium as Sea Water Cytophaga Agar.

Cultures were transferred every 10 to 15 days and were incubated at 10° C. to 15° C.

HISTORY OF THE ISOLATES

Cultures BS1 and BS2 were isolated from the Beaufort Sea composite sample which represented stations 1 to 30 at a depth of 50 to 100 meters. The initial enrichments were made on a sea water medium which contained chitin prepared from squid-pens.

Cultures BS3 and BS4 were isolated from the Beaufort Sea composite sample which represented stations 1 to 26 at a depth of 200 to 300 meters. Both of these isolates were obtained from chitin enrichments: BS3 from an inorganic medium supplemented with squid-pen chitin and BS4 from a sea water basal medium supplemented with squid-pen chitin.

Culture FO1 was isolated from Port Orchard Bay from a water sample obtained with the J-Z bacteriological water sampler. It represented a bottom water sample taken in 40 meters of water.

Culture FO2 was isolated from Port Orchard Bay from a core sample obtained with the Emery-Dietz gravity-type core sampler. The core length was 65 cm.; bottom was 42 meters.

Culture FO3 was isolated from Port Orchard Bay from a water sample collected with the J-Z bacteriological water sampler. The sample was obtained at 20 meters in 42 meters of water.

Culture FO4 was isolated from Port Orchard Bay from a mud sample obtained with the orange-peel bucket at a depth of 22 meters.

TABLE 1

SIZE AND SHAPE OF ISOLATES[1]

Culture Number	Size microns	Shape	Photomicrograph
BS1	0.5-0.7 x 2.5-6	rounded	
BS2	0.5-0.7 x 2.5-3	rounded	Plate I, fig. 3[2]
BS3	0.5-0.7 x 2.5-4	rounded	Plate I, fig. 1[3]
BS4	0.5-0.7 x 2.0-3	tapers	Plate I, fig. 2[2]
PO1	0.5-0.7 x 2.5-6	rounded	Plate II, fig. 1
PO2	0.4-0.6 x 6.0-20	rounded	Plate II, fig. 3[4]
PO3	0.4-0.6 x 6.0-20	rounded	Plate II, fig. 4[4]
PO4	0.4-0.6 x 2.5-6	rounded	Plate II, fig. 2

1 Measurements were made on 1-day-old to 2-day-old cultures which had been grown on petri plates containing a sea water 1 per cent agar base plus 0.05 per cent each of beef extract, yeast extract, and tryptone. Cultures were incubated at 10° C. to 15° C. Preparations were made by placing a small piece of agar on a glass slide and covering with a cover slip. A binocular, oil-immersion, phase microscope equipped with a calibrated micrometer was used.

2 Plate I, figures 2 and 3 demonstrate involution forms and microcysts, respectively.

3 Plate I, figure 1 shows involution forms.

4 Plate II, figures 3 and 4 show habit patterns obtained from impression smears of young cultures. Filaments are also present.

CHARACTERIZATION OF THE ISOLATES

Cytological and Morphological Investigations

Vegetative Cell

Variation in size of the vegetative cell among individuals of a clone is apparently a function of the conditions of growth. The age of a culture, the concentration of substrates, and the consistency of the substrate are some of the factors which govern size range.

Young cultures usually showed a predominance of long, slender rods and occasional filaments. Flexibility was marked. However, some cultures showed a considerable proportion of short rods and coccoidal elements at the earliest stages of development. Bizarre forms were present in aged cultures and could be confused with typical microcysts. Plate I, figure 2 demonstrates involution forms in contrast to microcysts shown in figures 3 and 4.

The effect of nutrient concentration and substrate consistency on vegetative cell morphology is discussed by Stanier (1947). An unfavorable medium favored the development of shorter cells which are suggestive of a small, nonmotile, Gram-negative, true bacterium. A 1 per cent agar base permitted the development of longer cell forms and also of filaments, whereas a 2 per cent agar base favored the development of shorter cells.

Consequently, prior to measurement of vegetative cells, the conditions of growth should be defined. Measurements of the eight isolates (see Table 1) were made from young cultures which had been grown on plates containing the Sea Water Cytophaga Agar medium.

TABLE 1

SIZE AND SHAPE OF ISOLATES[1]

Culture Number	Size microns	Shape	Photomicrograph
BS1	0.5-0.7 x 2.5-6	rounded	
BS2	0.5-0.7 x 2.5-3	rounded	Plate I, fig. 3[2]
BS3	0.5-0.7 x 2.5-4	rounded	Plate I, fig. 1[3]
BS4	0.5-0.7 x 2.0-3	tapers	Plate I, fig. 2[2]
PO1	0.5-0.7 x 2.5-6	rounded	Plate II, fig. 1
PO2	0.4-0.6 x 6.0-20	rounded	Plate II, fig. 3[4]
PO3	0.4-0.6 x 6.0-20	rounded	Plate II, fig. 4[4]
PO4	0.4-0.6 x 2.5-6	rounded	Plate II, fig. 2

1 Measurements were made on 1-day-old to 2-day-old cultures which had been grown on petri plates containing a sea water 1 per cent agar base plus 0.05 per cent each of beef extract, yeast extract, and tryptone. Cultures were incubated at 10° C. to 15° C. Preparations were made by placing a small piece of agar on a glass slide and covering with a cover slip. A binocular, oil-immersion, phase microscope equipped with a calibrated micrometer was used.

2 Plate I, figures 2 and 3 demonstrate involution forms and microcysts, respectively.

3 Plate I, figure 1 shows involution forms.

4 Plate II, figures 3 and 4 show habit patterns obtained from impression smears of young cultures. Filaments are also present.

Flexibility, Motility and Swarming

Degrees of flexibility of the vegetative cells are seen in young cultures. The photomicrographs shown in Plate IV, figures 1 and 3 demonstrate the numerous "S", "U", and "loop" configurations which may be present.

The rate of forward movement was measured. Some cells were observed to move at an average rate of 10 microns per minute. Stanier (1940) described myxobacterial motion as a slow, even gliding in one direction with none of the rapid directional changes associated with the motility of the flagellated organisms.

Typical mass swarming was observed for all cultures. Plate III demonstrates this characteristic. Figures 1 and 2 were taken approximately 1 hour apart. This short period excludes the possibility of outward colonial growth as a cause of colonial movement. Figure 3 demonstrates a typical myxobacterial edge; figure 4 shows the pathways formed in the agar by an agar-digester.

Microcysts and Fruiting Bodies

Conflicting opinions which arise concerning the presence or absence of microcysts could be due to their resemblance to ring forms. Comparison of Plate I, figure 2 with Plate I, figure 4 and with Plate IV, figure 4 shows the difference between ring forms and microcysts, respectively. The later photomicrographs should be compared to Speyer's (1953) picture of the microcysts of Sporocytophaga myxococcoides. Confirmation of macrocyst formation should show an increase in numbers with age, and, if possible, germination of the microcyst. The ability to form these

structures is greatly retarded with age of the culture and by serial transfer on artificial media.

Microcysts were formed by cultures F02, F03, and by all of the Beaufort Sea isolates.

Multiplication, Gram Reaction, and Granulation

Multiplication is transverse by constriction. Thus, the dividing end of the daughter cells may be tapered (see Plate I, figure 2).

All of the isolates were Gram-negative. Granulation was observed in the electron microscope (see Plate IV, figures 1 and 3).

Biochemical and Physiological Characterization

Biochemical and physiological characterization of members of the order Myxobacteriales has lagged far behind morphological studies. This section of the investigation represents an attempt to recognize differential biochemical and physiological characters of the isolates. Table 2 lists the composite results.

Nitrate Reduction

The isolates were grown in a sea water 0.2 per cent agar base which contained 0.1 per cent KNO_3 plus cytophaga supplement. Cultures were incubated at 10° C. to 15° C. for 8 days. The standard test for nitrite was used. Except for cultures BS2 and BS1, all of the isolates reduced nitrate to nitrite.

TABLE 2

GENERAL CHARACTERISTICS OF THE ISOLATES[1]

Test	BS1	BS2	BS3	BS4	PO1	PO2	PO3	PO4
Nitrate reduction	-	-	+	+	+	+	+	+
Gelatin liquefaction	-	-	-	-	+	+	+	+
Starch hydrolysis	-	-	-	-	+	-	-	+
Cellulose degradation	-	-	-	-	-	-	-	-
Chitin degradation	-	-	-	-	+	+	+	+
Agar liquefaction	-	-	-	-	-	-	-	+
Catalase	-	+	+	+	+	-	-	+
Cytochromes	-		+	+	+	-	-	+
Nadi test	-		+	+		-	-	+
Anaerobic growth	-	-	-	-	-	-	-	-

[1] Relative growth: -, no growth; +, growth.

Gelatin Liquefaction

Liquefaction of gelatin was observed in a medium which contained 15 grams of gelatin per 100 ml. sea water. Cultures were incubated for 8 days at 10° C. to 15° C. The four PO isolates liquefied gelatin; the BS isolates did not.

Starch Hydrolysis

Hydrolysis of starch was tested in a medium which contained: semi-solid agar, inorganic salts, an amino acid supplement of approximately 22 commercial amino acids, a vitamin supplement of approximately 12 commercial B vitamins, cytophaga suprlement, and soluble starch. Cultures were incubated at 10° C. to 15° C. for 10 days. Iodine was used as the test agent Cultures PO1 and PO4 were positive; the other cultures were negative.

Cellulose Utilization

The agar and starch were omitted from the starch hydrolysis test medium listed above, and cellulose was added in the form of filter paper or as a preparation obtained from the mats produced by Acetobacter xylinum (Dickerman and Starr, 1951). All cultures were negative.

Chitin Degradation

The medium used for this test contained the Sea Water Cytophaga Agar base supplemented with a suspension of chitin prepared from squid-pens. Dissolution of the suspended chitin was observed for the PO cultures only.

Agar Liquefaction

Culture PO4 liquefied agar readily on all media containing agar.

Sodium Chloride Tolerance

This test was performed in a liquid basal medium similar to that used for starch hydrolysis. Increasing concentrations of NaCl were added to the agar-free medium. Table 3 lists the results.

Carbohydrate Utilization

This test was performed in a complete medium supplemented with approximately 0.1 per cent of the sugars listed in Table 4. Utilization of the sugars was tested for by quantitative measurement of the amount disappearing, according to the method of Saifer et al (1941).

Catalase

Catalase was determined by the addition of 3 per cent hydrogen peroxide to a streak plate of the organism, and by the addition of a suspension of the organism to the peroxide. All of the isolates were catalase positive except for cultures PO2 and PO3.

Cytochromes

Cytochromes were determined with a hand spectrophotometer. A strong cytochrome c band was observed with cultures PO1, PO4, BS1, BS3, and BS4. No band could be detected for cultures PO2 and PO3.

TABLE 3

RELATIVE GROWTH IN THE PRESENCE OF NaCl

% NaCl	BS1	BS2	BS3	BS4	PO1	PO2[1]	PO3[1]	PO4
0	-	-	+-	+-	-	-	-	++
0.1	-	-	+	+	-	-	-	++
0.4	+	+	++	++	-	-	-	+++
1.0	++	+	++	++	-	-	-	+++
2.5	++	++	++	++	+	+	+	+++
4.0	++	++	++	++	+	+	+	+++
6.0	+-	-	+-	+-	-	-	-	+
8.0	-	-	-	-	-	-	-	+-
10.0	-	-	-	-	-	-	-	-

[1] PO2 and PO3: 0.2 per cent agar added to medium.

TABLE 4

UTILIZATION OF CARBOHYDRATES[1]

Sugar	BS1	BS2	BS3	BS4	PO1	PO2	PO3	PO4
No sugar	14/-	50/-	14/-	14/-	50/-	50/-	50/-	14/-
Arabinose	5/86	5/0 50/35	8/1	8/54	24/2 50/0	26/2	26/1	6/54
Xylose	14/0	50/0	8/3	8/0	24/2	26/3	26/1	5/11
Glucose	5/91	10/8 50/42	8/82	8/72	24/15 50/54	26/8	26/0	8/79
Fructose	5/80	50/0	5/5 14/50	8/3 14/52	24/0 50/12	26/2	26/0	8/65
Galactose	5/72	5/8 50/77	5/67	8/34	24/0 50/9	26/2	26/0	6/66
Lactose	5/91	50/0	8/87	8/64	50/0	26/0	26/0	8/74
Cellobiose	5/92	50/0	8/0	8/72	24/52	26/0	26/0	6/92
Sucrose	5/86	8/3	10/0	8/32	24/0	26/2	26/0	8/77

[1] Numbers indicate: days incubation/per cent sugar utilised.

Cytochrome Oxidase

Cytochrome oxidase was determined according to the method of Keilin (1929). A positive reaction was noted for cultures BS1, BS3, BS4, and PO4. Cultures PO2 and PO3 were negative.

Anaerobic Growth

Cultures were grown on complete media. Brewer anaerobe jars were used to obtain and maintain anaerobic conditions. The air was replaced by hydrogen and 5 per cent carbon dioxide. Anaerobiosis was determined using a methylene blue, alkali, and glucose indicator. Aerobic controls grew well within 5 days. No anaerobic growth was evident under the conditions of the experiments. Cultures which were incubated anaerobically for 8 days and which showed no signs of growth were subsequently incubated under aerobic conditions. Growth was evident in 5 days.

Nutritional Studies

The available literature which dealt with the nutritional requirements of members of the order Myxobacteriales revealed no distinctive characteristics common to the group as a whole. Apparently, heterogeneity of nutritional requirements is concurrent with their varied habitats and universal distribution as it is with other bacterial orders. An attempt was made in this part of the investigation to determine the minimal nutritive requirements of the isolates.

A synthetic inorganic base was developed which supported growth of all of the isolates upon the addition of cytophaga supplement

and carbohydrate. The synthetic inorganic base contained: (grams per 100 ml. solution) NaCl, 2.5; KCl, 0.1; $K_2HPO_4 \cdot 3H_2O$, 0.08; $MgSO_4 \cdot 7H_2O$, 0.04; $(NH_4)_2SO_4$, 0.001; and $FeCl_3 \cdot 6H_2O$, 0.0001. The complete medium had a final pH of 7.2 to 7.4. All experiments to be described were done in this pH range.

The isolates fell within four well-defined categories.

Cultures PO1 and PO4 were the least fastidious of the eight isolates. The former liquefied agar, whereas the latter did not. Table 5 summarizes the nutritive requirements of these isolates. Their minimal requirements were satisfied by an inorganic base plus a carbohydrate or by a synthetic mixture of amino acids in a nitrogen-free inorganic base.

Cultures PO2 and PO3 did not grow in liquid media which were rotated, and they grew poorly in liquid media which were incubated undisturbed. Growth was enhanced by the addition of a semi-solid agar to a synthetic medium. Table 6 summarizes the nutritional requirements of these isolates. It should be noted that the minimal requirements correspond to those of the previous group except for the effect of a semi-solid substrate on growth.

The growth requirements for cultures BS3 and BS4 were similar. They could be grown on a synthetic medium containing: amino acids, carbohydrate, and thiamin. Table 8 summarizes the results of several experiments which were designed to show the requirement for thiamin.

Cultures BS1 and BS2 both had a thiamin requirement which could be demonstrated in a complete medium containing: carbohydrate, cytophaga supplement, amino acids, and vitamin supplement minus thiamin.

TABLE 5

NUTRITIONAL REQUIREMENTS OF CULTURES P01 AND P04

	Additions to the Basal Medium[1]	Relative Growth P01	Relative Growth P04
1.	No additions	-	-
2.	$(NH_4)_2SO_4$, 0.0001 per cent	-	-
3.	$(NH_4)_2SO_4$, 0.1 per cent	-	-
4.	Glucose or lactose, 0.1 to 0.5 per cent	-	-
5.	2 + 4	-	-
6.	3 + 4		+[2]
7.	Amino acid supplement, 0.1 ml/100 ml[3]	-	-
8.	Amino acid supplement, 1.0 ml/100 ml	-	+4
9.	4 + 7	+-	++[5]
10.	4 + 8	+	+[5]
11.	Vitamin supplement, 1.0 ml/100 ml[3]	-	-
12.	2 + 4 + 7 + 11	+	++[6]

1 Basal medium, grams/100 ml: 2.5, NaCl; 0.1, KCl; 0.08, $K_2HPO_4 \cdot 3H_2O$; 0.04, $MgSO_4 \cdot 7H_2O$; and 0.0001, $FeCl_3 \cdot 6H_2O$.

2 High concentrations of inorganic nitrogen plus a carbohydrate supported growth.

3 Amino acid and vitamin supplements (see Table 7).

4 High concentrations of amino acids supported growth alone.

5 In the presence of carbohydrates, low concentrations of amino acids supported better growth than high concentrations.

6 Vitamins did not enhance growth.

TABLE 6

NUTRITIONAL REQUIREMENTS OF CULTURES PO2 AND PO3

	Additions to the Basal Medium[1]	Relative Growth
1.	No additions	-
2.	Casein hydrolysate, 0.005 per cent	+[2]
3.	Casein hydrolysate, 0.01 per cent	++
4.	Casein hydrolysate, 0.04 per cent	++
5.	Vitamin supplement, 1.0 ml/100 ml[5]	-
6.	2 + 5	+[3]
7.	Glucose, 0.5 per cent	-
8.	Amino acid supplement, 0.1 ml/100 ml[5]	-
9.	7 + 8	+-
10.	9 + cytophaga supplement, 1.0 ml/100 ml	+[4]

1 Basal medium, grams/100 ml: 2.5, NaCl; 0.1, KCl; 0.08, $K_2HPO_4 \cdot 3H_2O$; 0.04, $MgSO_4 \cdot 7H_2O$; 0.0001, $FeCl_3 \cdot 6H_2O$; 0.001, $(NH_4)_2SO_4$; and 0.2, agar.

2 Growth occurred in the inorganic base in the presence of casein hydrolysate.

3 Vitamin supplement did not enhance growth.

4 Cytophaga supplement enhanced growth in the presence of glucose and amino acids.

5 Amino acid and vitamin supplements (see Table 7).

TABLE 7

AMINO ACID SUPPLEMENT AND VITAMIN SUPPLEMENT

	per cent		per cent
L-arginine·HCl	0.03	Thiamin·HCl	0.05×10^{-3}
L-asparagine	0.03	Niacin	0.1×10^{-3}
L-histidine·HCl	0.01	Ca pantothenate	0.05×10^{-3}
DL-alpha alanine	0.03	Riboflavin	0.01×10^{-3}
L-hydroxyproline	0.0006	Pyridoxine·HCl	0.02×10^{-3}
L-leucine	0.005	Pyridoxamine·HCl	0.01×10^{-3}
DL-lysine·HCl	0.01	p-Amino-benzoic acid	0.01×10^{-3}
DL-methionine	0.006	Biotin	0.2×10^{-6}
DL-phenylalanine	0.004	Choline·HCl	1.0×10^{-3}
L-proline	0.004	i-Inositol	1.0×10^{-3}
DL-serine	0.01	Folic acid	0.1×10^{-6}
DL-threonine	0.01	Vitamin B-12	0.05×10^{-6}
L-tyrosine	0.004		
L-tryptophane	0.005		
DL-valine	0.005		
DL-isoleucine	0.005		
L-glutamic acid	0.05		
Glycine	0.05		

Stock solutions of the amino acid supplement were made in concentrated form and used at the rate of 5 ml/100 ml solution to achieve the final per cent of each indicated above.

Stock solutions of the vitamin supplement were made in concentrated form and used at the rate of 1.0 ml/100 ml solution to achieve the final per cent of each indicated above.

TABLE 8

NUTRITIONAL REQUIREMENTS OF CULTURES BS3 AND BS4

	Additions to the Basal Medium[1]	Relative Growth
1.	No additions	-
2.	Amino acid supplement, 0.1 to 0.5 ml/100 ml[6]	-
3.	Vitamin supplement, 1.0 ml/100 ml[6]	-
4.	Glucose or lactose, 0.5 per cent	-
5.	Cytophaga supplement, 1.0 ml/100 ml	+-
6.	2 + 3	+-
7.	2 + 3 + 4	++
8.	2 + 3 + 4 + 5	+++[2]
9.	7 minus thiamin	-[3]
10.	4 + thiamin, 0.1 mg/100 ml	+-[4]
11.	10 + amino acid supplement, 0.5 ml/100 ml	+[5]

1 Basal medium, grams/100 ml solution: 2.5, NaCl; 0.1, KCl; 0.08, $K_2HPO_4 \cdot 3H_2O$; 0.04, $MgSO_4 \cdot 7H_2O$; 0.001, $(NH_4)_2SO_4$; and 0.0001, $FeCl_3 \cdot 6H_2O$.

2 Addition of cytophaga supplement to the complete medium enhanced growth.

3 A medium deficient in thiamin would not support growth.

4 Thiamin supported growth in the presence of a carbohydrate.

5 Amino acids enhanced the thiamin effect.

6 Amino acid and vitamin supplements (see Table 7).

Addition of thiamin to this medium, which did not support growth, gave growth. Culture BS1 did not require the cytophaga supplement, whereas culture BS2 required the presence of cytophaga supplement. Table 9 summarizes these results.

Enzymatic Activity

A voluminous literature exists on the nature of carbohydrate utilization by bacteria, yeasts, plants, and mammalian tissues. Pathways for glucose degradation have been established and accepted. The purpose of this part of the investigation was to gain an insight into the problem of carbohydrate utilization by studying the enzyme systems of several of the isolates.

Organisms

The organisms used were selected from the eight isolates described in previous sections: BS3, BS4, and FO4.

Media Used

The following basal medium was used for growth of mass cultures: NaCl, 2.5 per cent; $K_2HPO_4 \cdot 3H_2O$, 0.08 per cent; $MgSO_4 \cdot 7H_2O$, 0.04 per cent; $(NH_4)_2SO_4$, 0.001 per cent; $FeCl_3 \cdot 6H_2O$, 0.0001 per cent; yeast extract, beef extract, and tryptone, 0.05 per cent each; amino acid supplement, 0.1 ml/100 ml; and vitamin supplement, 0.5 ml/100 ml. One-tenth per cent glucose or fructose was added to the basal medium.

TABLE 9

NUTRITIONAL REQUIREMENTS OF CULTURES BS1 AND BS2

	Additions to the Basal Medium[1]	Relative Growth BS1	Relative Growth BS2
1.	No additions	-	-
2.	Glucose or lactose, 0.5 per cent	-	-
3.	Amino acid supplement, 0.1 ml/100 ml[6]	-	-
4.	Vitamin supplement, 1.0 ml/100 ml[6]	-	-
5.	Cytophaga supplement, 1.0 ml/100 ml	-	-
6.	2 + 3 + 4	-	-
7.	2 + 3 + 4 + 5	+[2]	+
8.	7 minus thiamin	-[3]	-
9.	2 + 3 + 5 + thiamin 0.1 mg/100 ml	+[4]	+
10.	Thiamin, 0.1 mg/100 ml; lactose, 0.1 per cent; amino acid supplement, 0.5 ml/100 ml	+[5]	-
11.	5 + 10	+	+

1 Basal medium, grams/100 ml solution: 2.5, NaCl; 0.1, KCl; 0.08, $K_2HPO_4 \cdot 3H_2O$; 0.04, $MgSO_4 \cdot 7H_2O$; 0.001, $(NH_4)_2SO_4$; and 0.0001, $FeCl_3 \cdot 5H_2O$.

2 A complete medium supported growth of both isolates.

3 In the absence of thiamin, the complete medium did not support growth.

4 Thiamin could be supplemented for the cytophaga supplement.

5 Culture BS2 required cytophaga supplement in addition to thiamin as shown in Addition no. 11.

6 Amino acid and vitamin supplements (see Table 7).

Enzyme Preparation

Cells were harvested and washed in phosphate buffer (pH 6.8, M/20). Extracts were prepared by grinding equal parts of cells and levigated alumina. The mixture was suspended in five parts of phosphate buffer and spun down in the Sorvell centrifuge for 30 minutes at 10,000 rpm. The supernatant was used immediately or frozen prior to use.

Determination of Enzyme Systems

Hexokinase and fructokinase were determined according to the method of Klein (1951). Phosphohexoseisomerase was determined according to the method of Roe (1934) and Meyerhof (1933). Phosphofructokinase and aldolase were determined according to the method of Sibley and Lehninger (1949). Glucose-6-phosphate dehydrogenase and 6-phosphogluconic acid dehydrogenase were determined according to the method of Warburg et al (1935, 1937).

Results and Discussion of Enzyme Systems

The enzyme systems and the isolates which were tested are listed in Table 10.

An active system which catalyzed the reaction glucose (G) to glucose-6-phosphate (G-6-P) in the presence of adenosinetriphosphate (ATP) was found in both glucose and fructose grown cells of the three cultures tested. Table 4 lists the quantitative data which represents glucose and fructose utilization by these cultures. In addition, the enzyme system which catalyzed the reaction fructose (F) to fructose-1-phosphate (F-1-P) in the presence of ATP was tested for in glucose and fructose grown cells of cultures BSh and POh.

TABLE 10

ENZYME SYSTEMS INVESTIGATED

Substrate	Product	Enzyme	Cultures Tested		
G[1]	G-6-P[2]	Glucokinase[7]	BS4	PO4	BS3
F[3]	F-6-P[4]	Fructokinase[7]	BS4	PO4	
G-6-P	F-6-P	Phosphohexoseisomerase	BS4	PO4	BS3
F-6-P	F-1,6-P	Phosphofructokinase	BS4	PO4	
F-1,6-P	TP[5]	Aldolase	BS4	PO4	BS3
G-6-P	6-PGA[6]	G-6-P dehydrogenase	BS4	PO4	BS3
6-PGA	pyruvate	Splitting enzyme[9]	BS4	PO4	Ps. sp.[8]
6-PGA	(pentose)	6-PGA dehydrogenase	BS4		

1 G: glucose

2 G-6-P: glucose-6-phosphate

3 F: fructose

4 F-6-P: fructose-6-phosphate

5 TP: triose phosphate

6 6-PGA: 6-phosphogluconic acid

7 Glucokinase and fructokinase: both glucose and fructose grown cell-extracts were tested.

8 Ps. sp.: Pseudomonas saccharophila used as a control.

9 Splitting enzyme: Entner and Doudoroff (1952)

Evaluation of the results (see Table 11) indicated that several hexokinases may exist which differed mainly in their affinities for G and F. The investigations of Klein (1953) showed similar evidence for different hexokinases in Pseudomonas putrefaciens.

The next series of reactions which were investigated were those involved in the conversion of G-6-P to triosephosphates (TP). The three enzyme systems implicated were phosphohexoseisomerase, phosphofructokinase, and aldolase. They catalyzed the respective reactions: G-6-P to F-6-P to F-1,6-P to TP. Enzyme preparations which were tested gave positive evidence for the presence of these systems. Thus, the glycolytic enzyme systems as established for yeast, some bacteria, and mammalian tissue were present in all of the isolates tested. The steps involved the conversion of glucose and fructose to triose phosphates.

The observation that aerated cultures gave increased cell yields suggested the possibility of an alternate oxidative mechanism operating in carbohydrate utilization.

The enzyme system which catalyzes the reaction, G-6-P to 6-phosphogluconic acid (6-PGA), was studied. Culture BS4 was selected for study of the specificity of this reaction which was followed in the Beckman spectrophotometer (see Figure 1). The addition of triphosphopyridine nucleotide (TPN) to the reaction mixture which contained the enzyme preparation, G-6-P, and buffer gave the calculated change in optical density. Substitution of diphosphopyridine nucleotide (DPN) for TPN was ineffective. However, if TPN was added to the reaction mixture which contained the inactive DPN, a change in optical density was observed. Thus, the specificity of TPN for this enzyme system was established.

TABLE 11

GLUCOKINASE AND FRUCTOKINASE ACTIVITY OF PO4 AND BS4

Substrate[1]	Fructose[2] grown PO4	Glucose[3] grown PO4	Fructose[4] grown BS4	Glucose[5] grown BS4
Glucose, initial	26.5	28.0	28.3	34.0
Glucose, final	13.3	7.0	9.3	6.3
Fructose, initial	31.0	26.5	22.0	33.0
Fructose, final	31.0	20.3	10.0	21.8

[1] Substrate: numbers are micrograms per ml.

[2] Fructose grown PO4: strong glucokinase activity and no fructokinase activity.

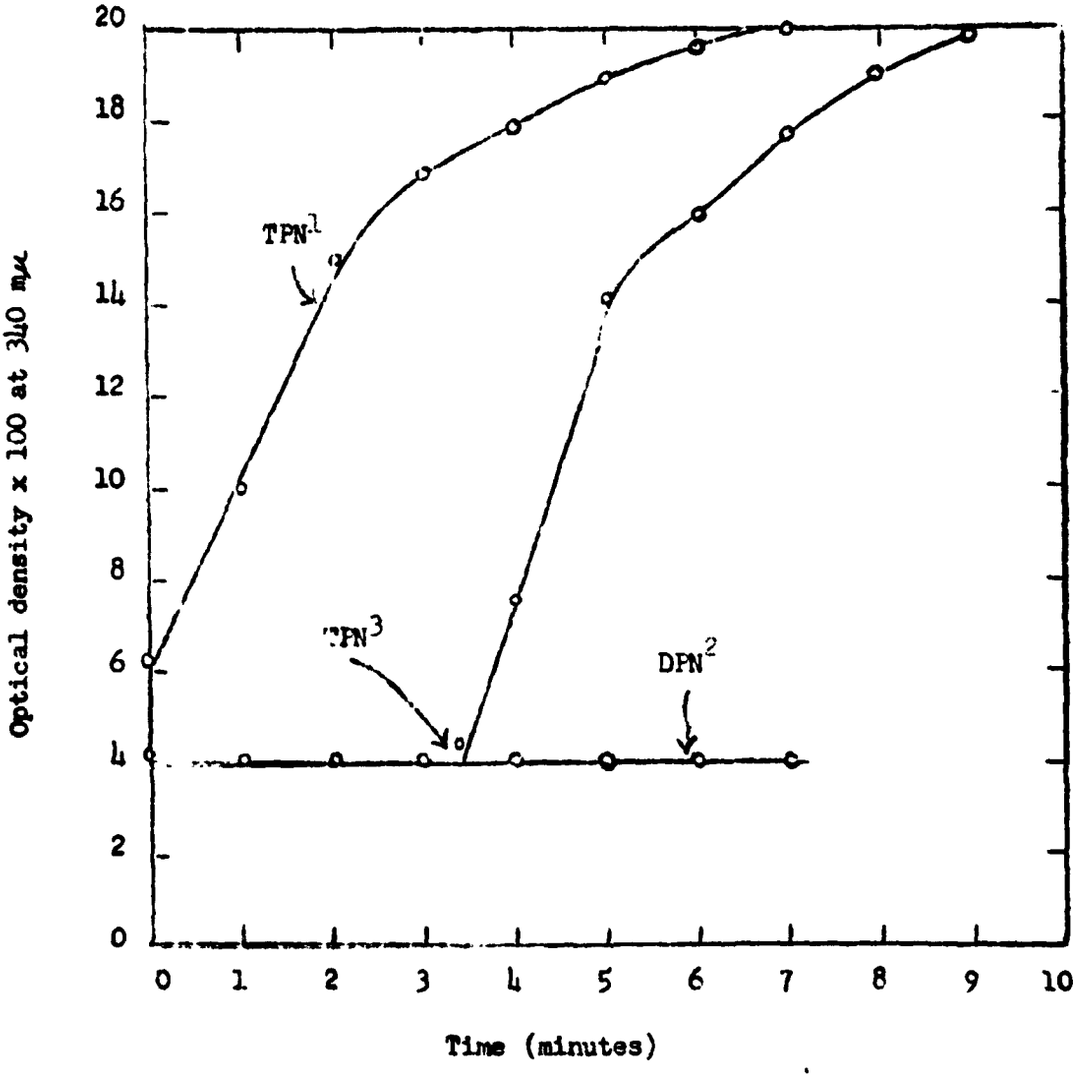
[3] Glucose grown PO4: strong glucokinase activity and weak fructokinase activity.

[4] Fructose grown BS4: strong glucokinase activity and strong fructokinase activity.

[5] Glucose grown BS4: strong glucokinase activity and weak fructokinase activity.

FIGURE 1

SPECIFICITY OF TPN FOR G-6-P DEHYDROGENASE OF CULTURE BS4



1 Beckman quartz-cuvettes were used. The reaction mixture contained in a total volume of 3.0 ml: 2.3 ml of Tris (hydroxymethyl) aminomethane buffer (M/20, pH 8.0), 0.1 ml of G-6-P (4.5 micromoles), 0.5 ml enzyme preparation, and 0.1 ml (100 micromoles) of TPN.

2 DPN was substituted for TPN as described in note 1. No change in optical density was noted.

3 TPN was added to the reaction mixture, described in note 2, at the time indicated.

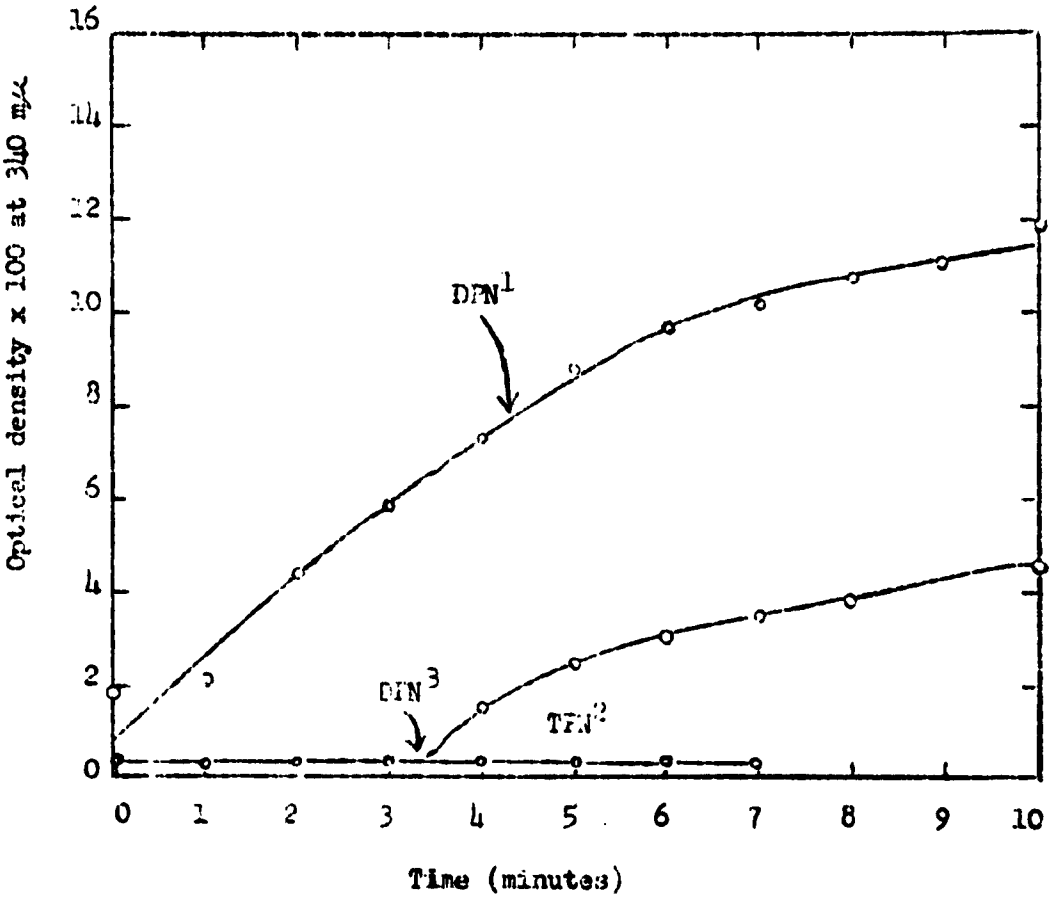
The next reaction studied was the step which involved the substrate 6-PGA (see Figure 2). It was shown to be DPN specific by methods similar to the G-6-P dehydrogenase reaction.

An attempt was made to determine the end product or products of the DPN specific 6-PGA dehydrogenase system. In order to avoid the addition of stoichiometric amounts of DPN to the reaction mixture which would enable identification of the product of the reaction, the reaction was coupled to a number of other systems which included: alcohol dehydrogenase, methylene blue, and yellow enzyme. Pentose could not be detected. The anaerobic split of 6-PGA to pyruvate did not work. A control which included Pseudomonas saccharophila gave a positive pyruvate test as expected.

In summary, the isolates tested had a glycolytic system which operated in the utilization of glucose and fructose to triosephosphates. In addition, culture BS4 possessed an oxidative mechanism which involved a TPN specific glucose-6-phosphate dehydrogenase and a DPN specific system which acted on 6-PGA. The product of the latter reaction has not been identified.

FIGURE 2

SPECIFICITY OF DPN FOR 6-PGA DEHYDROGENASE OF CULTURE BS4



1 Beckman quartz-cuvettes were used. The reaction mixture contained in a total volume of 3.0 ml: 2.6 ml of Tris (hydroxymethyl) aminomethane buffer (M/20, pH 8.0), 0.1 ml of 6-PGA (4.5 micromoles), 0.2 ml enzyme preparation, and 0.1 ml (100 micrograms) of DPN.

2 TPN was substituted for DPN as described in note 1. No change in optical density was noted.

3 DPN (0.05 ml, 50 micrograms or one-half the amount used for curve 1) was added to the reaction mixture described in note 2 at the time indicated.

SUMMARY

The over-all objective of this investigation was to attain a general understanding of the marine members of the order Myxobacteriales. The approach to this problem necessitated the isolation and characterization of representative species.

Samples were collected from the Port Orchard and Port Madison Bay regions of Washington by a variety of oceanographic techniques. Other samples included aged sea water from the Beaufort Sea which is located north of Alaska. The samples of muds, sea water, sediments, cores, and plankton tows were plated on a variety of media designed for the purpose. In addition, enrichment media were inoculated prior to plating. A large number of samples and isolation media were used in order to obtain as wide a variety of representative myxobacteria as possible. Myxobacteria were isolated from the bay samples by direct plating; the aged Beaufort Sea samples required initial enrichment techniques prior to plating.

Myxobacteria were isolated from most of the samples. Eight types were selected for characterization of properties. They were representative of the myxobacteria observed during the course of this study. They were chosen on the basis of general properties which included: colonial characteristics and pigmentation, microscopic cellular differences, action on agar, and by the presence or absence of microcysts.

Characterization of the eight isolates was based on studies which included: cytological and morphological investigations, biochemical and physiological differentiation, nutritive requirements, and enzymatic activity.

Cytological and morphological investigations showed typical myxobacterial characteristics for all of the isolates. In general, they were slender, Gram-negative rods which possessed a considerable degree of flexibility and gliding motility. Colonial swarming was evident. Microcysts were present in some.

Biochemical and physiological differentiation was based on such tests as: presence or absence of catalase, cytochromes, and cytochrome oxidase; reduction of nitrates to nitrites; action on chitin, cellulose, starch, gelatin, agar, and eight sugars; tolerance to sodium chloride; and anaerobic growth. One species liquefied agar, none used cellulose, and all were aerobic. The other characters differed with the species.

Minimal nutritive requirements of the isolates ranged from those species which could be grown on a mineral nitrogen base plus a carbohydrate to those species which, in addition, required amino acids and thiamin, and to those species which required chemically undefined growth factors.

Enzymatic studies on three of the isolates showed a typical glycolytic scheme. In addition, an oxidative system was present. The oxidative scheme of one of the isolates involved a triphosphopyridine nucleotide-specific glucose-6-phosphate dehydrogenase and a diphosphopyridine nucleotide-specific system which acted on 6-phosphogluconic acid. The end product of the latter reaction was not determined.

On the basis of these differential characters, five different species were recognized among the eight isolates studied. Taxonomic positions for these species are recommended. The three marine members

of the genus Cytophaga which had been described previously are considered also. Pending further investigation of fruiting body formation by freshly isolated strains, the eight myxobacteria studied can be classified in the following manner (Breed et al, 1948):

Family I. Cytophagaceae

Genus I. Cytophaga

Diagnosis: Flexible, sometimes pointed rods, showing a creeping motility. No fruiting bodies or spores(microcysts) formed.

I. From soil.
II. From sea water.
  A. Liquefies agar.
    1. Starch utilized.
      a. Cellulose utilized.....1. Cytophaga krzemieniewskae
      b. Cellulose not utilized.....2. Culture P04
    2. Starch not utilized.
      a. Nitrates reduced.
        1) Gelatin liquefied.....3. Cytophaga diffluens
      b. Nitrates not reduced.
        1) Gelatin not liquefied.....4. Cytophaga sensitiva
  B. Agar not liquefied.....5. Culture P01

Family V. Myxococcaceae

Genus IV. Sporocytophaga

Diagnosis: Microcysts formed loosely in masses of slime among the vegetative cells. Fruiting bodies absent.

I. Catalase negative.....1. Cultures P02 and P03
II. Catalase positive.
  A. Nitrates reduced.....2. Cultures BS3 and BS4
  B. Nitrates not reduced.....3. Cultures BS1 and BS2

PHOTOMICROGRAPHS

Plate I

Figure 1. Culture BS3. Three days old. Impression mount. Stained with basic fuchsin. Note involution forms and microcysts. 1600x.

Figure 2. Culture BS4. Three days old. Impression mount. Stained with basic fuchsin. Note involution forms and cells in the process of division by constriction. 1600x.

Figure 3. Culture BS2. Three days old. Impression mount. Stained with basic fuchsin. Round bodies are microcysts. 1600x.

Figure 4. Culture PC3. Three days old. Impression mount. Stained with crystal violet. Shows microcysts. 1600x.

Plate II

Figure 1. Culture PC1. Four days old. Impression mount. Stained with basic fuchsin. Shows slender vegetative cells. 1500x.

Figure 2. Culture PC4. Four days old. Impression mount Stained with basic fuchsin. Shows vegetative cells. 1500x.

Figure 3. Culture PC2. Four days old. Impression mount Stained with crystal violet. Shows typical habit pattern. 1500x.

Figure 4. Culture PC3. Two days old. Impression mount. Stained with basic fuchsin. Shows habit pattern. 1600x.

Plate III

Figures 1 and 2. Culture FC3. Two days old. A colony edge. Pictures were taken 1 hour apart, directly from petri plate. Note swarming. 140x.

Figure 3. Culture FC2. Seven days old. A typical colony edge. 160x.

Figure 4. Culture FC4. Two days old. Note pathways formed by this agar digester. 160x.

PHOTOMICROGRAPHS (continued)

Plate IV

Figure 1. Culture P01. Three days old. Electron photomicrograph. 6000x.

Figure 2. Culture P04. Three days old. Electron photomicrograph. 6000x.

Figure 3. Culture P02. Three days old. Electron photomicrograph. 6000x.

Figure 4. Culture P03. Three days old. Impression mount. Stained with crystal violet. Shows microcysts. 1500x.

Plate I

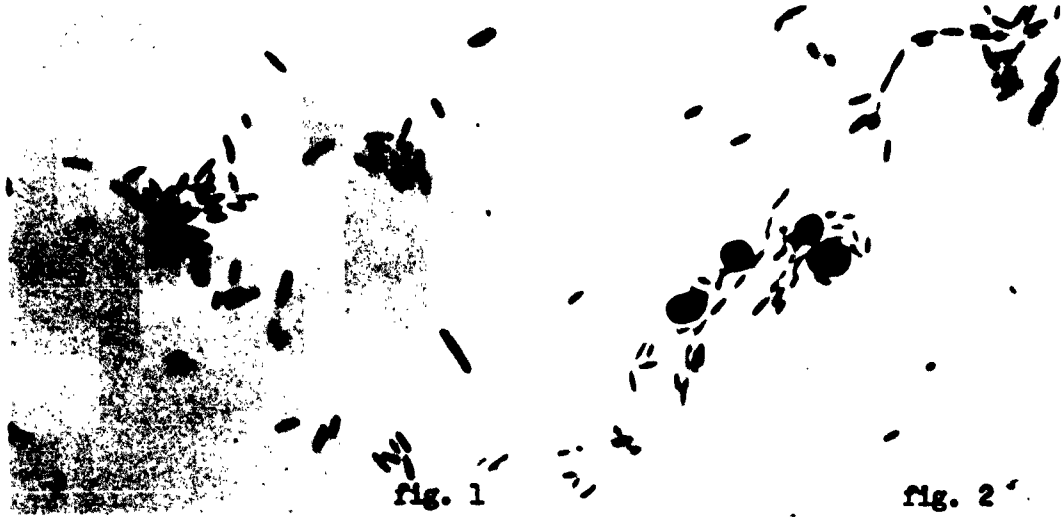


fig. 1 fig. 2

fig. 3 fig. 4

Plate II

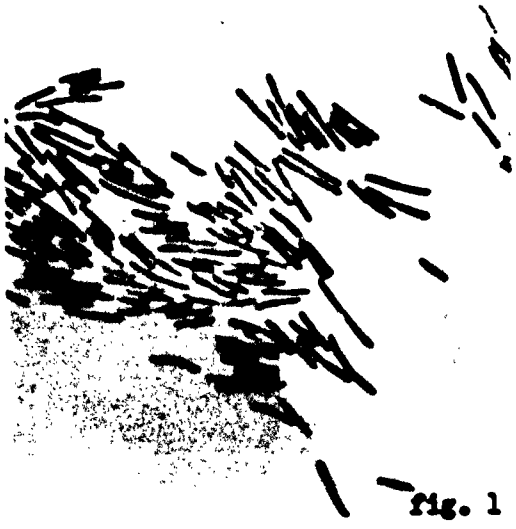


Fig. 1

Fig. 2

Fig. 4

Plate III

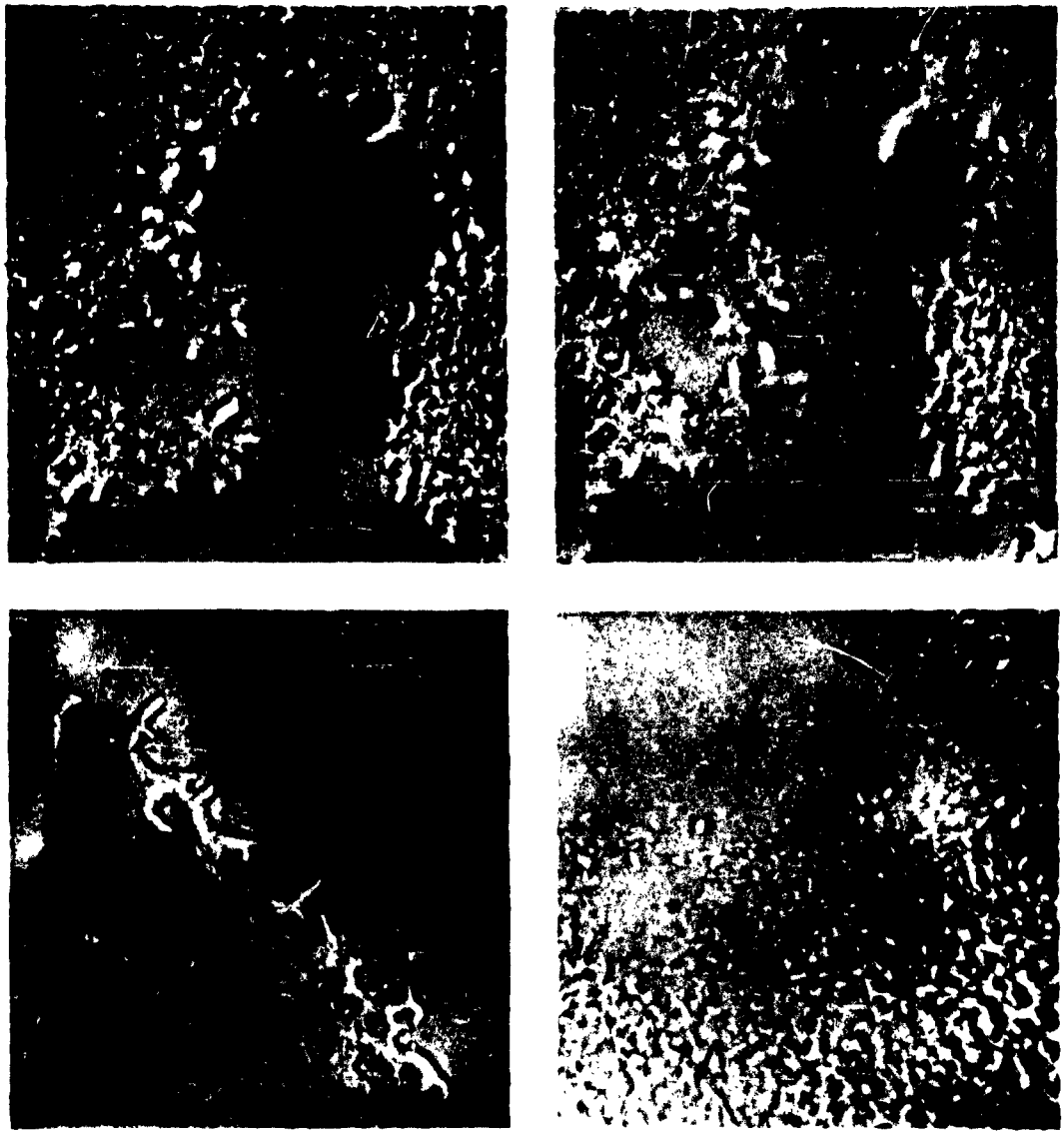


Plate IV

fig. 3

fig. 4

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